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Role of apoptosis in uranyl acetate-induced acute renal failure and acquired resistance to uranyl acetate

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Role of apoptosis in uranyl acetate-induced acute renal failure and acquired resistance to uranyl acetate.

Background. We have previously reported that animals recovering from uranyl acetate (UA)-induced acute renal failure (ARF) were resistant to subsequent insult. Recent evidence suggests that apoptosis participates in tubular damage. We investigated the role of apoptosis in UA-induced ARF and attenuation of ARF in acquired resistance to UA in rats.

Methods. ARF was induced by an intravenous injection of UA (5 mg/kg) in rats. Rats of group 1 were injected with UA and followed for 28 days. Group 2 rats were injected with a second dose of UA (5 mg/kg) 14 days after the first injection and were followed for 14 days. All rats received an intraperitoneal injection of bromodeoxyuridine (BrdU) one hour before sacrifice. Using kidneys, histologic examination and immunohistochemical detection of proliferating cell nuclear antigen (PCNA), BrdU, Bcl-2, and Bax were performed. To detect apoptosis, electron microscopy, analysis of DNA fragmentation, and the TUNEL methods were adopted.

Results. UA increased the number of damaged renal tubules and serum creatinine, which peaked at 5 days in group 1, but both returned to baseline values by 14 days. Apoptosis was confirmed by electron microscopy and the "ladder" pattern of DNA fragments on gel electrophoresis. The number of apoptotic tubular cells evaluated by the TUNEL method showed two peaks at days 5 and 14 in group 1. The second peak of TUNEL-positive cells was preceded by an increased number of BrdU-positive nuclei, PCNA-positive nuclei, and total number of tubular epithelial cells. Renal damage after the second UA injection was markedly reduced. The peak number of apoptotic cells in group 2 was significantly less than that in group 1.

Conclusions. Two peak levels of apoptotic cells occurred in UA-induced ARF. The first peak might play a role in UA-induced tubular damage, while the second one might represent the removal of excess regenerating cells during the recovery phase. Modulation of apoptotic cell death might be involved in the acquired resistance to rechallenge injury by UA.

Although the term acute renal failure (ARF) has been used as a synonym of acute tubular necrosis, it has recently been confirmed that another form of tubular cell death, apoptosis, contributes to tubular damage in ARF [1–5]. Apoptosis has been described in both in vivo and in vitro studies of tubular cell damage caused by ischemia [1–5] and nephrotoxins, such as cisplatin [5–7], ochratoxin A [8], cadmium [9], as well as gentamicin [10]. However, the pathophysiological role of apoptotic tubular cell death in functional and morphological changes in ARF remains to be clarified. Shimizu and Yamanaka demonstrated that ischemia is followed by two peaks of apoptotic cell population in rats [1]. They speculated that the second peak of apoptotic cells contributes to the removal of excessively increased regenerating cells during the recovery phase. Nakajima et al noticed that uninephrectomy-induced attenuation of tubular damage was associated with the reduction of the first peak of apoptotic cell death in ischemic ARF, suggesting that the first peak of apoptosis may contribute to the ischemia-induced tubular damage [3]. To our knowledge, however, the time course of apoptotic cell death and its pathophysiological role have never been examined in nephrotoxin-induced ARF.

Animals recovering from ARF are reported to be resistant to the subsequent insult with nephrotoxins, including uranyl acetate (UA) [11–17], and this phenomenon is termed acquired resistance. In a series of studies, we have demonstrated in UA-induced ARF that the resistance to ARF is not due to altered renal hemodynamics [11], loss of response to vasoactive substances [12, 13], or less accumulation of UA in renal tissues [14], suggesting that renal tubular cells that recover from ARF must become resistant to a rechallenge by UA. Acquired resistance has also been observed in cultured porcine proximal tubular cells (LLC-PK_1) [15]. In these cells, acquired resistance to UA was induced by pretreatment with heat stress and abolished by agents that inhibit the production of heat shock proteins (HSPs) [15]. Recently, a close association between the expression of HSP73 and acquired resistance to UA was observed in UA-induced ARF in rats [16]. HSPs are known to be cell protective [15, 16], but at the same time attenuate apoptotic cell death [18]. These findings suggest that apoptotic cell

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death might play an important role in UA-induced ARF and that the acquired resistance to UA might be caused by a milder degree of apoptotic cell death.

In the present study, we investigated whether the administration of UA induces apoptosis in the rat kidney, and then evaluated the role of apoptosis in the development of UA-induced ARF and in the acquired resistance to UA. We also examined the expression of apoptosis-related proteins, Bcl-2 and Bax [19–21], in this model.

METHODS

Induction of uranyl acetate-induced acute renal failure and acquired resistance to acute renal failure

A total of 83 male Sprague-Dawley rats weighing 240 to 260 g (SLC Co., Shizuoka, Japan) was used in the present study. The rats had free access to standard rat diet and drinking water. The animals were divided into two groups. In group 1, rats received a single intravenous injection of 5 mg/kg of UA, and 3 or 5 rats each were sacrificed before and at 1, 3, 5, 7, 9, 14, 15, 17, 19, 21, and 28 days after UA injection. Group 2 (acquired resistance model) rats received 5 mg/kg of UA, followed by a second injection of 5 mg/kg of UA, 14 days after the first injection, and five rats each were sacrificed at 1, 3, 5, 7, and 14 days after the second UA injection. UA was dissolved in saline and administered through the dorsal penile vein.

The serum creatinine (S_{Cr}) level was measured by the enzymatic method (Mizuho Medy, Saga, Japan). For determination of DNA synthesis, all rats were injected intraperitoneally with 40 mg/kg 5-bromo-2′-deoxyuridine (BrdU; Sigma Chemicals Co., St. Louis, MO, USA) one hour before sacrifice, and the kidneys were removed under intraperitoneal pentobarbital sodium (30 mg/kg) anesthesia for histologic examination and DNA analysis. The kidney samples for DNA analysis were quickly frozen in liquid nitrogen.

Histologic examination

Kidney tissues were fixed in 10% neutral-buffered formalin solution, dehydrated in graded alcohol, and then embedded in paraffin. For detection of tubular damage, 3 μm thick sections were stained with periodic acid-Schiff reagent. The degree of tubular damage in the outer medulla, where the tubular damage is most evident in UA-induced ARF, was assessed at ×400 magnification using 20 randomly selected fields. Injury was categorized into one of five scores using the following criteria: (0) normal; (1) desquamation of tubular epithelial cells and areas of focal granulovacuolar epithelial cell degeneration that were <25% of tubules; (2) 25 to 50% of tubules; (3) 50 to 75% of tubules; and (4) tissues with 75 to 100% of the area being affected. The mean score in each rat represented the average score of all 20 fields. To assess the total number of tubular cells, reflecting the balance of tubular cell apoptosis and tubular cell proliferation, we counted the number of epithelial cells in 100 cross-sections of the proximal tubules in the outer medulla and calculated the number of cells per proximal tubule. To minimize observer bias, morphometric examination was performed without the knowledge of the group from which the tissue was obtained.

Electron microscopic detection of apoptotic cells

Small portions of renal tissues from one or two rats sacrificed at each time interval were fixed in 2% glutaraldehyde in 0.1 mol/L phosphate buffer (pH 7.4) at 4°C for two hours, postfixed in 1% osmium tetroxide resolved in the same buffer for one hour, and then embedded in Epon. Apoptotic cells were identified among proximal tubular cells by their morphologic features in toluidine blue-stained semi-thin sections examined by light microscopy. Ultra-thin sections that contained the suspected apoptotic cells were counterstained with UA and lead citrate and then examined under an electron microscope (JEM-1220; JEOL, Tokyo, Japan).

Analysis of DNA fragmentation

DNA fragmentation analysis was performed as described previously by Smith et al, with minor modifications [22]. Frozen kidney tissues from three rats sacrificed at each time interval were minced well with a razor blade. In the next step, 2 μL of the pasty tissue were suspended in 20 μL lysis buffer [50 mmol/L tris(hydroxymethyl) aminomethane hydrochloride (pH 7.8), 10 mmol/L disodium ethylenediaminetetraacetic acid (EDTA), and 0.5% sodium N-lauroyl sarcosinate] and incubated with 2 μL proteinase K (10 mg/dL) for 90 minutes at 50°C, followed by incubation with 1 μL ribonuclease A (10 mg/dL) for 30 minutes at 50°C. The resulting DNA preparations were electrophoresed through a 2% agarose gel containing ethidium bromide. DNA fragmentation was visualized and photographed under ultraviolet illumination.

Semiquantitative measurement of apoptosis

To visualize the 3′-OH ends of DNA fragments in apoptotic cells, the paraffin sections were prepared by the terminal deoxynucleotidyl transferase (TdT) technique (TUNEL method) using the ApopTag *In situ* Apoptosis Detection Kit (Oncor, Inc., Gaithersburg, MD, USA) [23]. The kit uses the 3′-OH end-extension of fragmented DNA using TdT and a detection system based on an antidigoxigenin antibody conjugated with peroxidase. Tissues were prepared according to the protocol provided by the manufacturer, with a sham incubation (without TdT) performed for every tissue specimen tested. In addition, a positive control consisting of a section of normal kidney incubated with 80 U/mL of DNAse I (Sigma) for one hour at room temperature was also pro-

cessed in all studies. The number of TUNEL-positive nuclei in the tubular epithelium of the outer medulla was counted in a blinded fashion using a light microscope at ×400 magnification. More than 20 microscopic fields per section were examined in tissues of each animal.

Immunohistochemical detection of cell proliferation, Bcl-2, and Bax expression

Cell proliferation was evaluated by two methods using a monoclonal antibody (mAb) against proliferating cell nuclear antigen (PCNA) and mAb against BrdU containing nuclease for DNA. For immunohistochemical detection of PCNA, BrdU, Bcl-2, and Bax proteins, paraffin sections were treated with 0.3% hydrogen peroxide in phosphate-buffered saline (PBS) for 30 minutes at room temperature. For the detection of BrdU, sections were pretreated with 0.1% proteinase type XXIV (Sigma) in 0.01 mol/L PBS for five minutes at 37°C before treatment with hydrogen peroxide [24]. After incubation of the sections with 20% normal goat serum for 10 minutes at room temperature, they were incubated with the mAb against PCNA (Oncogene Science, Uniondale, NY, USA) for 30 minutes at 37°C, mAb against BrdU containing nuclease for DNA (Amersham International plc, Buckinghamshire, UK) for one hour at 37°C or mAb against Bax (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) overnight at 4°C and then incubated with peroxidase-conjugated goat antimouse IgG (Nichirei, Tokyo, Japan) for 10 minutes at room temperature. For detection of Bcl-2, sections were incubated with rabbit polyclonal Ab against Bcl-2 (Santa Cruz) and then incubated with peroxidase-conjugated goat antirabbit IgG (Nichirei) for 10 minutes at room temperature. Finally, the reaction products were visualized using hydrogen peroxide containing 3,3′-diaminobenzidine in 0.05 mol/L tris buffer.

Proliferating cell nuclear antigen (PCNA)-positive nuclei and BrdU-positive nuclei in the tubular epithelium of the outer medulla were counted using a light microscope at ×400 magnification. The intensity of Bcl-2 and Bax staining in tubular epithelial cells in the outer medulla was scored using the following criteria: (0) negative staining; (1) weakly staining found in normal control rats; (2) intermediate staining between 1 and 3; and (3) strongly positive staining. The mean score was determined based on examination of more than 1000 tubular epithelial cells in each experimental animal in a blinded fashion.

For immunohistochemical controls, the primary antibodies were omitted or replaced with the corresponding animal's normal serum (antirabbit IgG for Bcl-2 and antimouse IgG2b for Bax).

Statistical analysis

Data were presented as mean ± SD. For comparisons of group data and intergroup data, one-way analysis of variance (ANOVA) with Fisher's protected least significant difference was employed by using the Stat View program. A P value of less than 0.05 denoted a statistically significant difference.

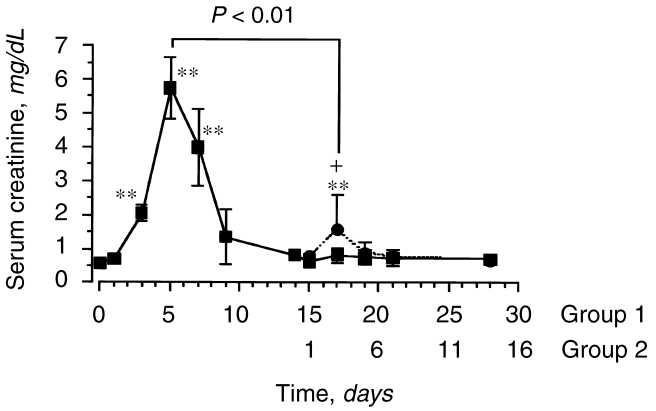


Fig. 1. Serial changes in serum creatinine (S_{Cr}) during the study. The first injection of UA in group 1 induced a significant increase in S_{Cr}, which reached a peak concentration on day 5. The increase in S_{Cr} following the second injection of UA in group 2 (●) peaked on day 3, but the peak level was significantly less than that in group 1 (■). $^{**}P <$ 0.01 vs. before UA injection; $+P < 0.05$ vs. 17 days after UA injection in group 1.

RESULTS

Serum creatinine

In group 1, S_{Cr} significantly increased as early as three days after the first injection of UA ($P < 0.01$ vs. level before induction of ARF), reached a peak value at five days ($P < 0.01$ vs. level before induction), and then returned to normal by 14 days (Fig. 1). In group 2, the second injection of UA, 14 days after the first injection, induced a significant increase in S_{Cr} three days later ($P <$ 0.01 vs. before induction and $P < 0.05$ vs. day 17 in group 1; Fig. 1). The peak concentration of S_{Cr} in group 2 was significantly lower than in group 1 ($P < 0.01$).

Tubular damage

In group 1, tubular damage assessed in periodic acid-Schiff–stained sections was evident three days after the first UA injection and was most pronounced at day 5 (Fig. 2A). In group 2, the tubular damage following the second UA was markedly reduced (Fig. 2B) compared with that observed in group 1 (Fig. 2A). Semiquantitative analysis of tubular damage showed that the score of tubular damage significantly increased at three days in group 1 ($P < 0.01$ vs. before induction), reached a peak level at five days ($P < 0.01$ vs. before induction), and then returned to the baseline by 14 days (Fig. 3). In group 2, the score of tubular damage significantly increased as early as three days after the second UA injection ($P <$ 0.01 vs. before induction or 5 days in group 1), but nor-

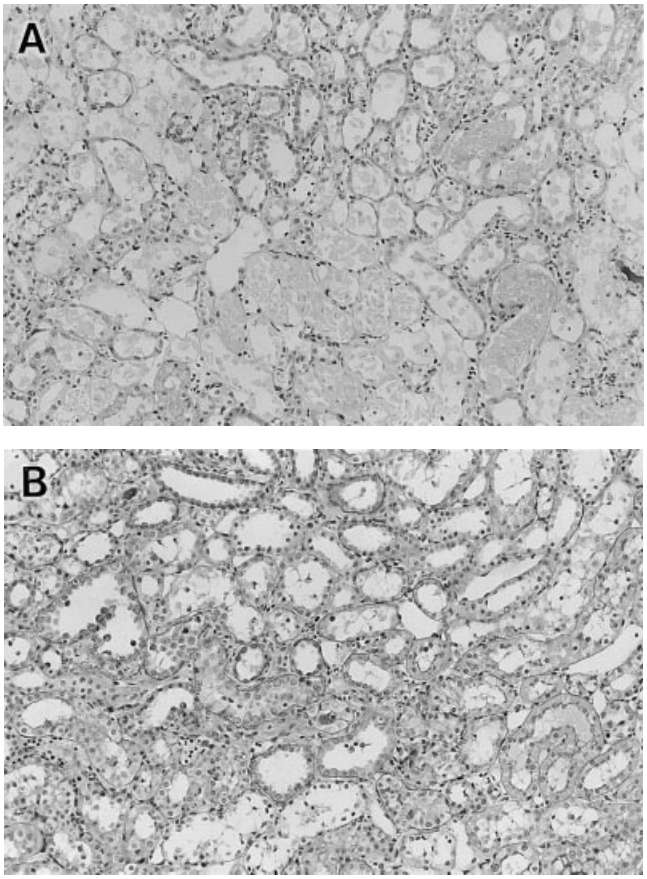


Fig. 2. Photomicrographs of periodic acid-Schiff–stained sections showing proximal tubular injury in the outer medulla. The tubular damage was most severe at day 5 in group 1 (*A*) and at day 3 in group 2 (*B*).

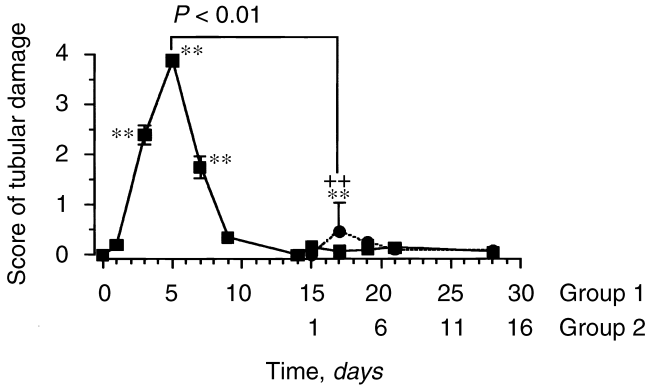


Fig. 3. Time course of tubular damage in the outer medulla. Tubular damage following the second injection of UA in group 2 (●) was significantly less than that following the first injection in group 1 (■). $^{**}P < 0.01$ vs. before UA injection; $++P < 0.01$ vs. the same day after UA injection in group 1.

malized by seven days (Fig. 3). The peak value of the score in this group was significantly lower than in group 1 ($P < 0.01$).

Apoptosis

Tubular cells with morphologic features of apoptosis, as determined by light microscopy, were detected as early as three days after the first UA injection and thereafter in group 1, and also after the second UA injection in group 2. However, the number of apoptotic cells in group 1 was low except that at five days. Electron microscopic examination showed typical apoptotic cells (Fig. 4), with clumping of the nuclear chromatin and its accumulation at the nuclear rim. However, the integrity of the plasma membrane and cell organelles was preserved in these cells. Some apoptotic cells were found in the tubular lumen as casts (Fig. 4B), but apoptotic cells anchored to the tubular basement membrane were also observed (Fig. 4A).

Agarose gel electrophoresis showed a typical "ladder" pattern of DNA fragments in kidney samples obtained five days after the first UA in group 1. No such pattern was observed at baseline (before induction of ARF; Fig. 5). The "ladder" pattern was equivocal at day 1 in group 1 and days 3 and 7 in group 2 (Fig. 5).

For a semiquantitative analysis of apoptosis, we counted the number of TUNEL-positive tubular cells. The number of such cells showed two peaks; it increased three days after the first UA injection in group 1 ($P < 0.05$ vs. before induction of ARF), reached a peak value at day 5 ($P < 0.01$ vs. before induction; Fig. 6A), and then decreased to baseline level by day 9 (Fig. 7), increased again to a peak level at day 14 ($P < 0.01$ vs. before induction), but decreased to baseline by day 21 (Fig. 7). In group 2, the number of TUNEL-positive cells increased one day after the second injection, remained at maximum levels between days 3 and 7 (Fig. 6B), and failed to return to baseline by day 14 (Fig. 7). The peak number of TUNEL-positive cells in group 2 was significantly lower than that at day 5 in group 1 ($P < 0.01$ vs. after second injection; Fig. 7).

Cell proliferation

The number of tubular epithelial cells per tubular cross sections significantly decreased in group 1, bottomed at day 5, and then increased over the baseline level and peaked at day 9, but gradually returned to a normal level by day 21 (Fig. 8A). The number of tubular epithelial cells in group 2 was higher than that in group 1 until seven days after the second UA injection (Fig. 8A).

The number of BrdU-labeled tubular epithelial nuclei significantly increased in group 1, with a peak value noted five days after the first UA injection ($P < 0.01$ vs. before induction of ARF; Fig. 9A), which then returned to baseline by day 14 (Fig. 8B). In group 2, the number of BrdU-labeled nuclei slightly but significantly increased three days after the second UA injection ($P < 0.01$ vs. before

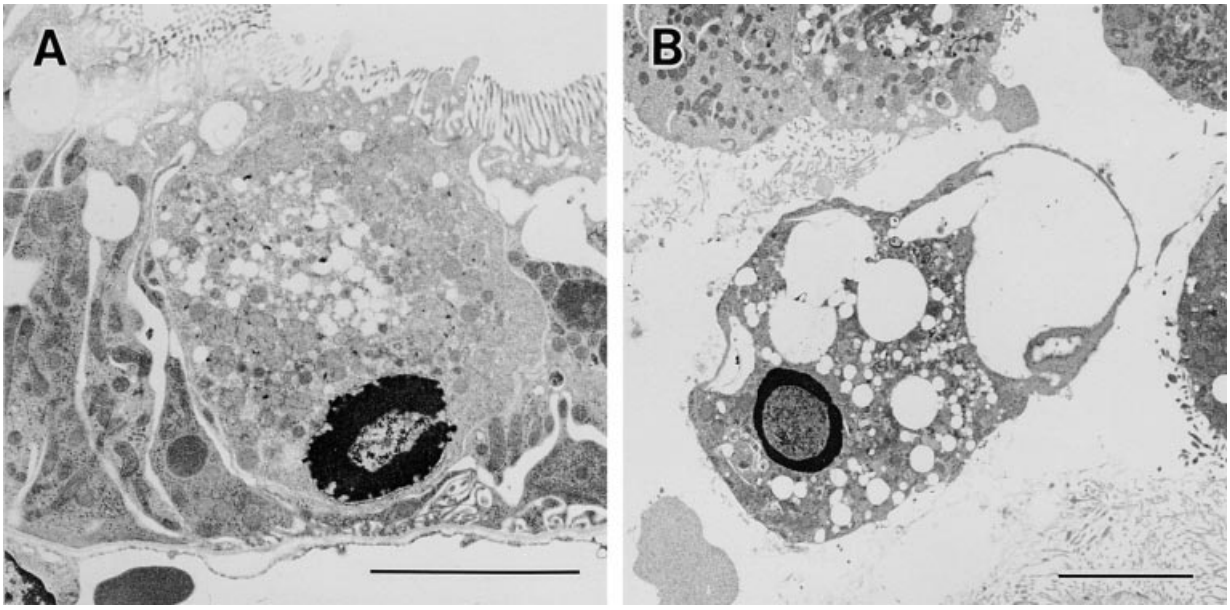


Fig. 4. Ultrastructure of apoptotic cells. Proximal tubular cells with condensed chromatin and relatively intact cell organelles (*A* and *B*) were detected five days after the first UA injection, indicating apoptotic cells. Some apoptotic cells were in the tubular lumen (B), but apoptotic cells anchored (A) to the tubular basement membrane were also observed. Bar = 5 μm.

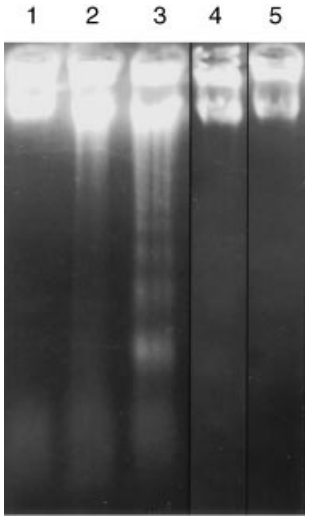


Fig. 5. Analysis of DNA by agarose gel electrophoresis. The "ladder" pattern of DNA fragments was observed five days after the first UA injection in group 1 (lane 3), but not before the induction of ARF (lane 1), one day after the first UA injection in group 1 (lane 2) or three and seven days after the second UA injection in group 2 (lanes 4 and 5, respectively).

induction or day 5 in group 1; Fig. 9B) and then returned to normal by day 7 (Fig. 8B).

The number of PCNA-positive tubular epithelial nuclei in group 1 significantly increased as early as three days after the first UA injection and reached a peak value at day 5 (Fig. 9C), but returned to normal by day 15 (Fig. 8C). In group 2, the number of PCNA-positive nuclei significantly increased, peaked three days after

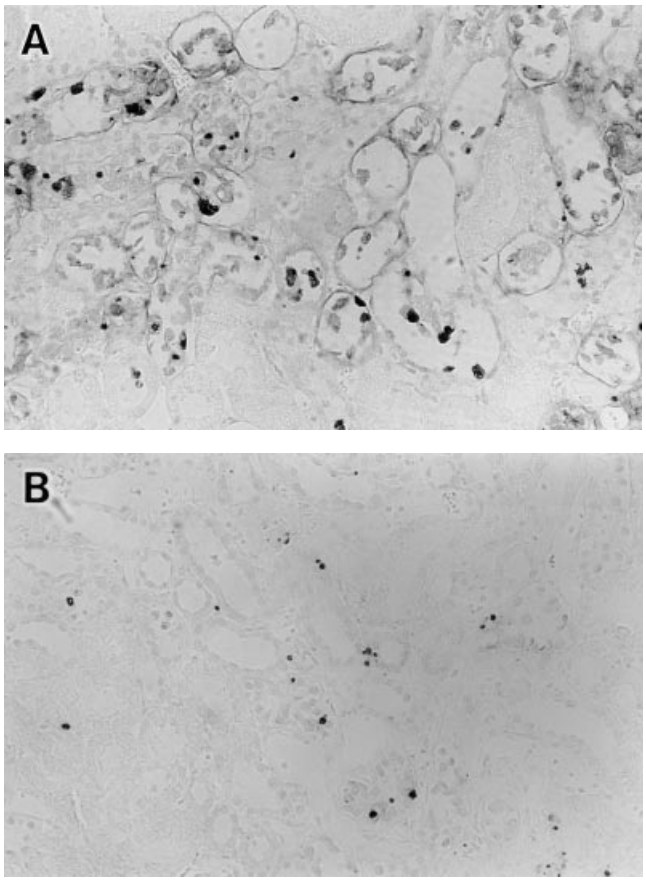


Fig. 6. Immunohistochemical findings of TUNEL-positive cells at five days after the first UA injection in group 1 (*A*) and seven days after the second injection in group 2 (*B*).

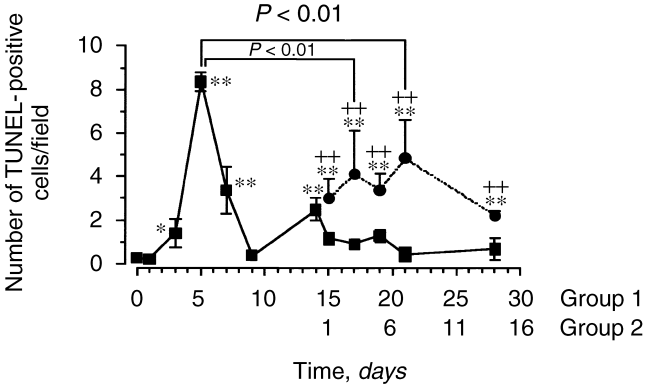


Fig. 7. Serial changes in the number of TUNEL-positive cells in the outer medulla. The number of TUNEL-positive cells in group 1 (■) increased as early as day 3, peaked at day 5, then normalized by day 21. In group 2 (●), the number of TUNEL-positive cells further increased one day after the second UA injection and did not return to a normal level during the experimental period. The number of TUNEL-positive cells at any days examined in group 2 was significantly less than that at day 5 in group 1. $*P < 0.05$ vs. before UA injection; $**P < 0.01$ vs. before UA injection; $++P < 0.01$ vs. the same day after UA injection in group 1.

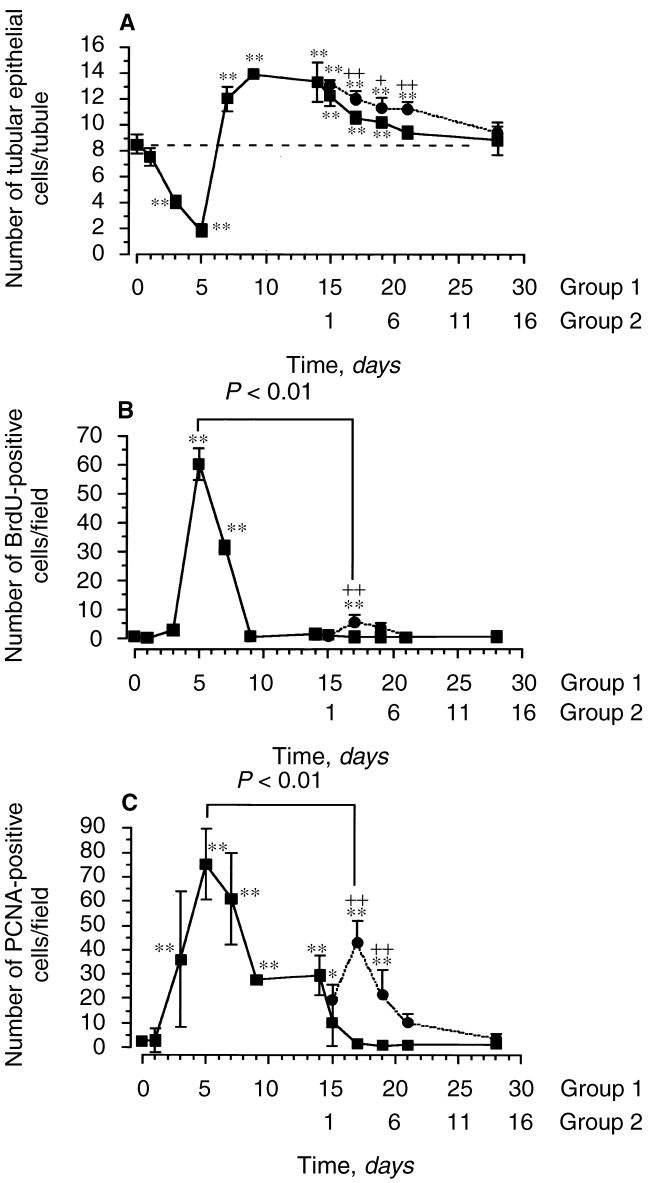


Fig. 8. Actual number of tubular epithelial cells per cross-sections of proximal tubule (*A*) and serial changes in the number of BrdU-labeled (*B*) and PCNA-labeled nuclei (*C*) in the outer medulla. Symbols are: (■) group 1, (●) group 2. $*P < 0.05$ vs. before UA injection; $**P < 0.01$ vs. before UA injection; $+P < 0.05$ vs. the same day after UA injection in group 1; $++P < 0.01$ vs. the same day after the first UA injection in group 1.

the second UA injection (Fig. 9D), and returned to baseline by day 7 (Fig. 8C).

Bcl-2 and Bax protein expression

Before the induction of ARF, a weak staining for Bcl-2 and Bax protein was constitutively present in the tubules (Fig. 10A) compared with that in histologic controls, which showed a completely negative staining (Fig. 10B). The constitutive staining in normal rats was judged as criteria 1 (normal staining). The score of Bcl-2–positive cells in group 1 remained at the control level or even decreased at least until nine days after the first UA injection (Fig. 10C), markedly increased at day 14 (Fig. 10G), and then returned to the control level by day 17 (Fig. 11A). In five days, Bcl-2–positive cells decreased in proximal tubular epithelial cells and increased in cast or necrotic cells in the tubular lumen (Fig. 11C). When primary antibody against Bcl-2 was replaced with antirabbit IgG, no significant staining was observed at day 5 in group 1 (Fig. 11D). In group 2, the score of Bcl-2–positive cells peaked one day after the second UA injection and decreased by day 5. A second peak of the score of Bcl-2-positive cells was observed seven days after the second UA (Fig. 11A).

In group 1, the score of Bax-positive cells slightly increased three days after UA injection ($P < 0.05$ vs. before induction; Fig. 10E), and significantly decreased at day 5 (Fig. 11B), and then gradually increased to a peak value at day 14 (Fig. 10H), but returned to the baseline level by day 17 (Fig. 11B). In three days, Bax-positive cells increased in not only proximal tubular epithelial cells, but also cast or necrotic cells in tubular lumen (Fig. 11E). When primary antibody against Bax was replaced with antimouse IgG2b, no significant staining was observed at day 3 in group 1 (Fig. 11F). In group 2, the score of Bax-positive cells reached a peak level as early as day 1 following the second UA, but then decreased. The score of Bax-positive cells in group 2 was always higher than normal rats throughout the observation period (Fig. 11B).

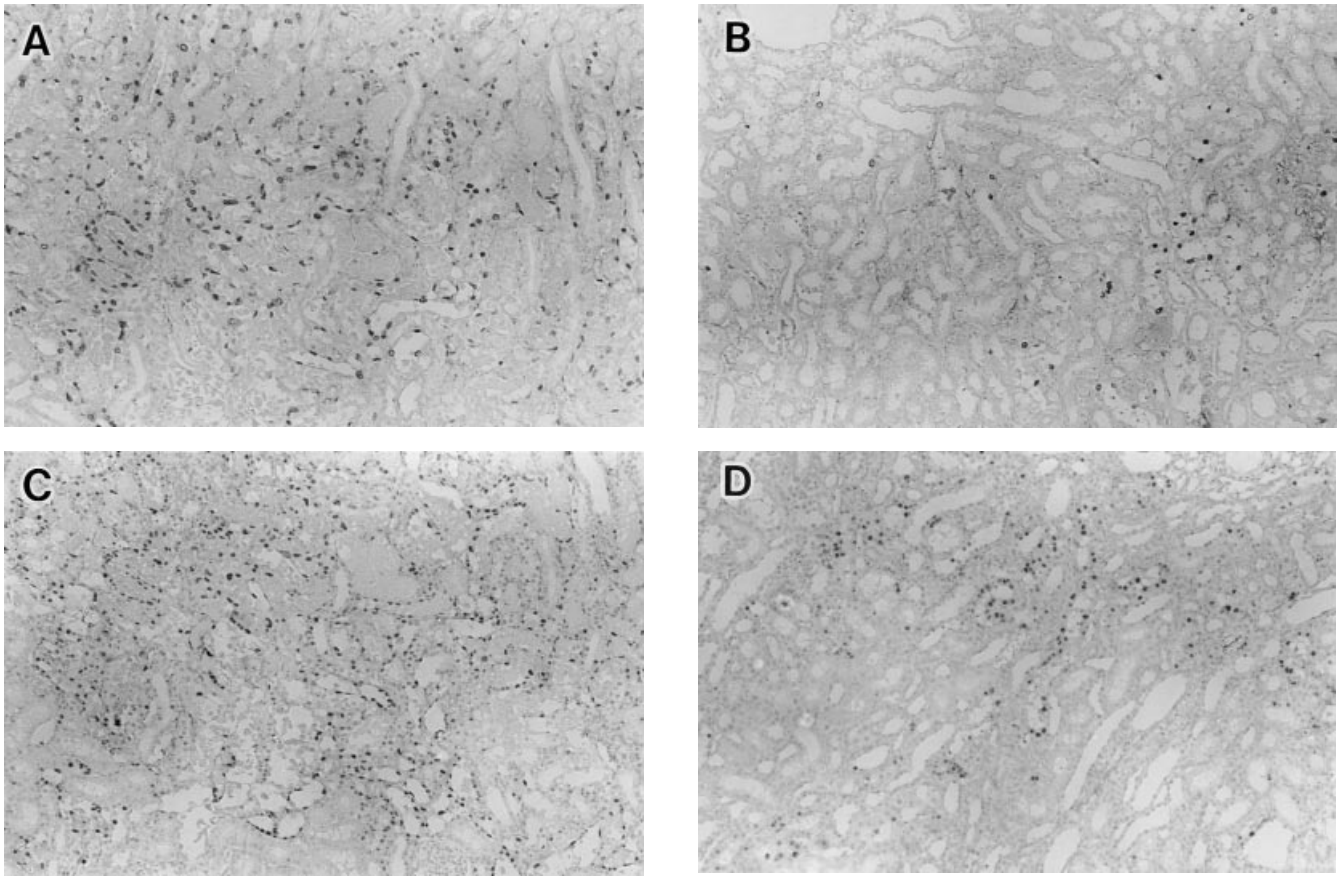


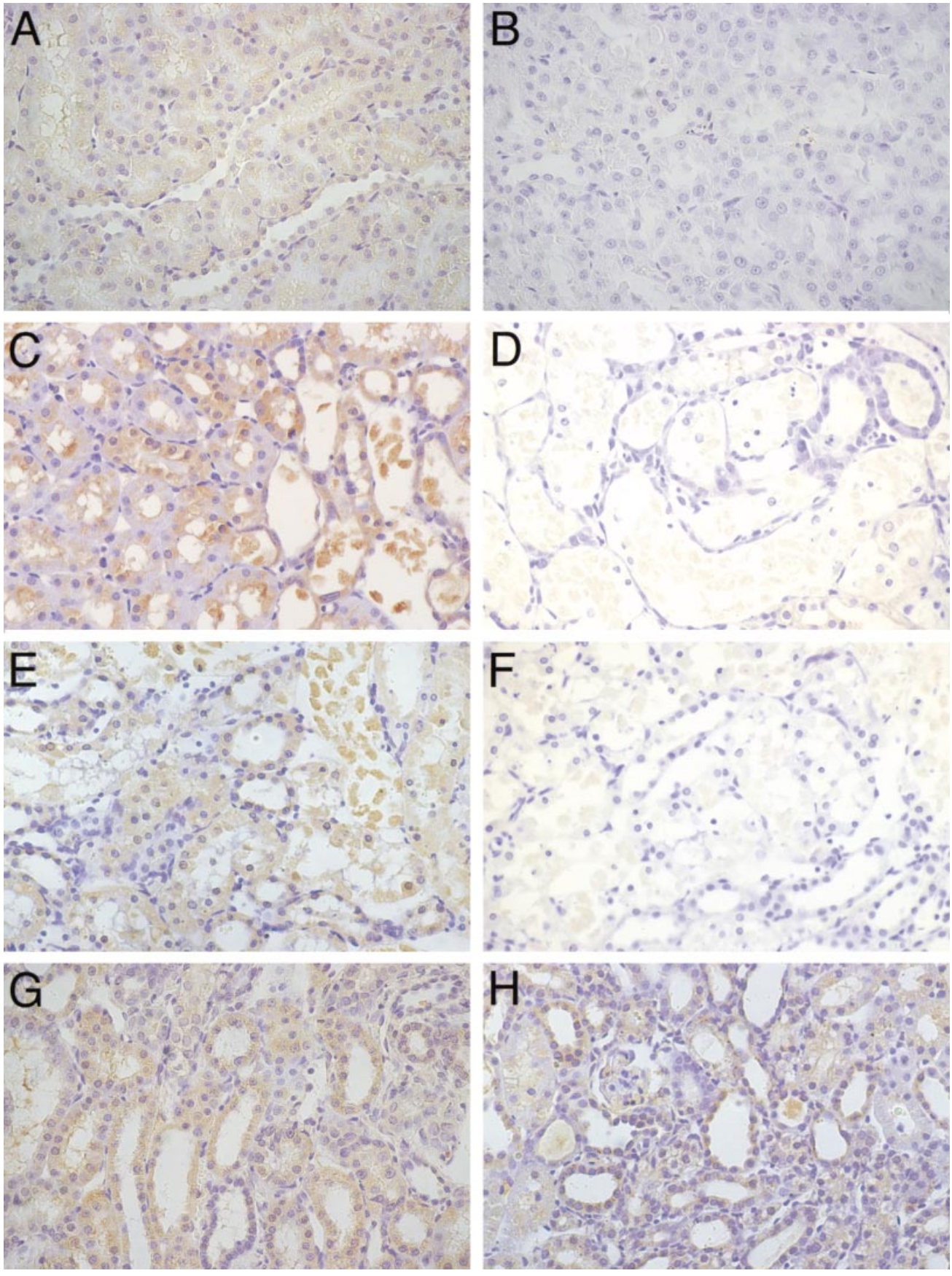
Fig. 9. Immunohistochemical findings of BrdU-positive (*A* and *B*) and PCNA-positive cells (*C* and *D*). The peak number of BrdU-positive cells was observed at five days in group 1 (A) and at three days after the second UA in group 2 (B). The number of PCNA-positive cells in group 1 peaked at day 5 (C) and at day 3 in group 2 (D).

DISCUSSION

In the present study, we examined the development of apoptosis and semiquantitated the serial changes in the number of apoptotic cells in UA-induced ARF. We also evaluated the role of apoptosis in the development of UA-induced ARF and in the attenuation of ARF in the acquired resistance to UA in rats. Our data showed the development of acute but transient renal failure following the first UA injection manifested by an increase in serum creatinine and tubular damage, which reached peak levels at day 5 but returned to basal level by day 14. In contrast, the second injection of UA induced a less pronounced increase in serum creatinine and a milder degree of tubular damage compared with the changes observed after the first injection. These functional and morphological changes were similar to those reported previously [17].

To investigate the role of apoptosis in ARF, we first demonstrated the presence of typical apoptotic tubular cells by electron microscopy (Fig. 4), and found that these apoptotic cells were most frequent at day 5 of UA-induced ARF. In addition, the "ladder" pattern of DNA fragments was observed on agarose gel electrophoresis at day 5 (Fig. 5). These findings suggest that apoptosis occurs in UA-induced ARF and that the peak of apoptosis coincides with the most prominent tubular damage. Since analysis of DNA fragments used here was not sensitive enough to quantitate apoptosis, we used the

Fig. 10. Immunohistochemical findings of Bcl-2 (*A*, *C*, and *G*) and Bax-positive cells (*E*, *H*). Weak staining for Bcl-2 over the tubules could be constitutively seen in the kidney before induction of ARF (A). On the other hand, histochemical control using PBS instead of the primary antibody for Bcl-2 or Bax showed totally negative staining in the kidney before induction of ARF (*B*). The lowest level of Bcl-2–positive cells was observed at day 5 in group 1 (C) and the peak level at day 14 in group 1 (G). Bax-positive cells were slightly increased at day 3 in group 1 (E) and peaked at day 14 in group 1 (H). When primary antibodies were replaced with the corresponding animal's normal IgG, antirabbit IgG for Bcl-2 at day 5 in group 1 (*D*) and antimouse IgG2b for Bax at day 3 in group 1 (*F*), no significant staining was observed.


A
B
C
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E
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H

TUNEL method for semiquantitative analysis of apoptotic cells. The highest number of TUNEL-positive cells was found five days after the first UA injection, coinciding with the detection of apoptotic cells by electron microscopy and "ladder" pattern of DNA fragments, strongly indicating that the number of TUNEL-positive cells represents the degree of apoptosis.

The serial changes in the number of TUNEL-positive nuclei during the first nine days in group 1 (Fig. 7) matched those of serum creatinine (Fig. 1) and the score of tubular damage (Fig. 3). In addition, the number of TUNEL-positive cells after the rechallenge with UA in group 2 (Fig. 7) diminished in a manner similar to the attenuated increase in serum creatinine (Fig. 1) and the degree of tubular damage (Fig. 3). These findings indicate that apoptotic cell death plays a role in UA-induced tubular damage and renal dysfunction.

The second injection of UA in group 2 also induced a significant increase in TUNEL-positive cells (Fig. 7). However, the number of these cells in the second challenge was significantly less than after the first injection. Based on the above conclusion that the first peak of apoptosis contributes to the development of tubular damage, the milder form of apoptosis following the second UA might reflect an acquired resistance to UA-induced ARF.

Exact roles of apoptosis, however, remain to be clarified. In general, apoptotic cell death affects surrounding tissues less and minimizes further tissue damage than necrotic cell death. If necrotic cell death induced by UA is replaced by apoptosis in rechallenged insults, the apoptotic cell death might be considered a protective process. However, we do not have any evidence that less necrotic cell death in rechallenged injury was associated with the change of cell death from necrotic cell death to apoptotic cell death. In UA-induced ARF, necrotic cell death was also observed in kidneys, and necrosis was reduced in rechallenged insult. Therefore, it is possible that necrotic cell death rather than apoptosis plays an important role in the development of tissue damage in UA-induced ARF and that the less kidney damage in rechallenged injury was caused mainly by less necrotic cell death. In this study, however, we did not evaluate the degree of necrosis, since we do not have a method to count the number of necrotic tubular cells in vivo. Furthermore, the number of TUNEL-positive cells reflects only a part of apoptotic cells because the apoptotic cells are removed by macrophages and are shed into tubular lumen in short time. For these reasons, we could not evaluate the relative number of apoptotic and necrotic cells in ARF at this moment. Despite these limitations, the close correlation between the number of TUNEL-positive cells and tubular damage suggests that there is a possibility that apoptotic cell death plays a key role in the development of UA-induced ARF. In accordance with this hypothesis,

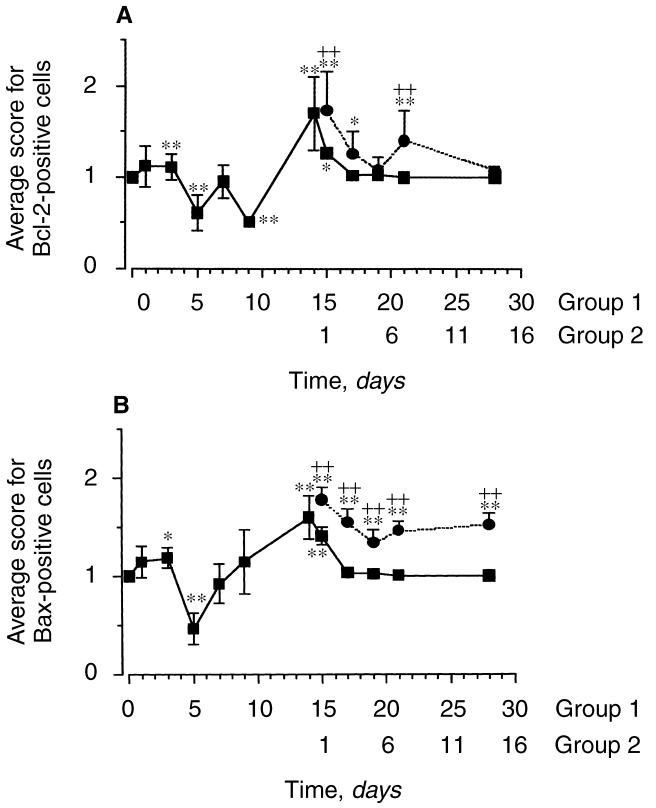

Fig. 11. Average score for Bcl-2–positive cells (*A*) and Bax-positive cells (*B*) in the outer medulla in group 1 (■) and group 2 (●) rats. $^{*}P < 0.05$ vs. before UA injection; $^{**}P < 0.01$ vs. before UA injection; $^{++}P < 0.01$ vs. the same day after the UA injection in group 1.

two previous reports indicate a major role of apoptosis in ischemic injury. Yoshimura et al quantitated the necrotic and apoptotic cell death of cultured cells during ischemia and found that apoptotic cell death was four times greater than necrotic cell death after 72 hours of ischemia [25]. In an in vivo study, Kajstura et al examined necrotic cell death of myocyte using myosin mAb, and concluded that apoptosis, not necrosis, is the major form of myocardial damage produced by occlusion of a major epicardial coronary artery [26]. Further studies are needed to conclude the exact roles of apoptotic cell death in the development of UA-induced ARF. The severity of kidney tissue damage induced by ischemic or toxic insults is determined by both the severity of the insult and the exposure time to it. In contrast, the development of apoptotic or necrotic cell death may be determined mainly by the severity of the insult rather than the duration of exposure time. Lieberthal et al showed that severe insult, such as a high concentration of cisplatin or a severe degree of adenosine 5′-triphosphate (ATP) depletion, induced cell necrosis and that mild insult resulted in apoptosis [7, 27]. In their data, a long-time exposure to mild insult, such as a low concentration of cisplatin or mild ATP depletion, can cause more cell damage than

short-time exposure to severe insults. These data suggest that in some situations severe kidney damage might be induced by the induction of apoptosis alone.

Our results showed that the increase in PCNA-positive nuclei in group 2 was relatively higher than BrdU-positive nuclei (Fig. 8 B, C). PCNA was initially considered as a marker of cell proliferation [28], but more recent studies have demonstrated that PCNA is expressed not only in proliferating cells but also in nonproliferating cells in the G1-phase of DNA repair [29–31]. On the other hand, BrdU incorporation correlates with the S-phase of the cell cycle since only BrdU incorporated in the nuclei of the S-phase can be recognized [32]. Thus, the increased number of PCNA-positive nuclei after the second UA injection partially reflects an increased population of cells in the G1-phase rather than in the S-phase. Consistent with this conclusion, Mizuno et al demonstrated that HSP73, which participates in the repair of damaged DNA [33], increases during the recovery phase in UA-induced ARF [16]. It is also possible that intensification of the process of DNA repair also contributed to a lower level of apoptosis and tubular damage in the second UA challenge.

The present study demonstrated a return of renal morphology and function to the normal status by 14 days after the first UA injection. However, a second peak of TUNEL-positive nuclei was observed 14 days after UA injection (Fig. 7) when no additional insult was imposed. It is unlikely that the second peak of TUNEL-positive cells was caused by UA toxicity itself. Interestingly, the second peak of TUNEL-positive cells was preceded by a significant increase in tubular cell proliferation (evaluated by the number of BrdU-positive nuclei and PCNA-positive nuclei) and number of tubular cells (Fig. 8). The increase in TUNEL-positive cells during this phase was associated with a decrease in the number of tubular epithelial cells. These findings suggest that apoptotic cell death in this phase might be caused by the programmed removal of excess cells after cell hyperplasia during the recovery from UA-induced tubular damage. In the ischemia-induced ARF model, Shimizu and Yamanaka demonstrated a similar biphasic pattern of apoptosis and a second peak of apoptosis followed an increase in cell proliferation and total tubular cells, suggesting that apoptosis in the late phase may participate in the programmed removal of excess epithelial cells during recovery from tubular damage [1].

Apoptosis is regulated by several molecules, including Bcl-2 and Bax proteins. Bcl-2 expression inhibits apoptosis through heterodimerization [19, 20], while Bax accelerates apoptosis [21]. In the present study, Bcl-2 protein expression is diminished on days 5 and 9 and then increased and peaked 14 days after the first UA injection compared with normal controls (Fig. 11A). On the other hand, the expression of Bax protein transiently increased at day 3, decreased below the baseline level at day 5, and then returned to the baseline level (Fig. 11B). The increased expression of Bax and the decreased expression of Bcl-2 during the development of ARF suggests a role for Bcl-2/Bax regulation in the development of apoptosis. The overall Bcl-2 and Bax protein expression, however, was not prominent; therefore, further studies are needed to obtain conclusive data about the role of Bcl-2/Bax regulation in the development of tubular damage. On the other hand, both Bcl-2 and Bax proteins were markedly increased in the same group at day 14 (Fig. 11), when the late phase of apoptosis occurred, suggesting that Bcl-2/Bax regulation may participate mainly in the programmed removal of excessively proliferated epithelial cells during the recovery phase.

In summary, in a rat model we demonstrate that an injection of a bolus dose of UA induces two peaks of apoptotic cells in the kidney tubules. The first peak indicates that apoptosis plays a role in the development of tubular damage in UA-induced ARF. The second peak of apoptosis, probably regulated by Bcl-2/Bax, represents the removal of excessively proliferated cells. Our results also demonstrate that a second injection of UA produced a less severe form of tubular damage, which is associated with a lower degree of apoptosis relative to the first injection. The reduced level of apoptosis may contribute to the acquired resistance to UA.

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REFERENCES

1. Shimizu A, Yamanaka N: Apoptosis and cell desquamation in repair process of ischemic tubular necrosis. *Virchows Arch B* 64:171–180, 1993
2. Schumer M, Colombel MC, Sawczuk IS, Gobé G, Connor J, O'Toole KM, Olsson CA, Wise GJ, Buttyan R: Morphologic, biochemical, and molecular evidence of apoptosis during the reperfusion phase after brief periods of renal ischemia. *Am J Pathol* 140:831–838, 1992
3. Nakajima T, Miyaji T, Kato A, Ikegaya N, Yamamoto T, Hishida A: Uninephrectomy reduces apoptotic cell death and enhances renal tubular cell regeneration in ischemic acute renal failure in rats. *Am J Physiol* 271:F846–F853, 1996
4. Basile DP, Liapis H, Hammerman MR: Expression of bcl-2 and bax in regenerating rat renal tubules following ischemic injury. *Am J Physiol* 41:F640–F647, 1997
5. Lieberthal W, Levine JS: Mechanisms of apoptosis and its potential role in renal tubular epithelial cell injury. *Am J Physiol* 271:F477–F488, 1996
6. Takeda M, Fukuoka K, Endou H: Cisplatin-induced apoptosis in mouse proximal tubular cell line. *Contrib Nephrol* 118:24–28, 1996
7. Lieberthal W, Triaca V, Levine JS: Mechanisms of death induced by cisplatin in proximal tubular epithelial cells: Apoptosis vs. necrosis. *Am J Physiol* 270:F700–F708, 1996

8. Seegers JC, Böhmer LH, Kruger MC, Lottering ML, De Kock M: A comparative study of ochratoxin A-induced apoptosis in hamster kidney and Hela cells. *Toxicol Appl Pharmacol* 129:1–11, 1994
9. Tanimoto A, Hamada T, Koide O: Cell death and regeneration of renal proximal tubular cells in rats with subchronic cadmium intoxication. *Toxicol Pathol* 21:341–352, 1993
10. Nuwen EJ, Verstrepen WA, Buyssens N, Zhu MQ, De Broe ME: Hyperplasia, hypertrophy, and phenotypic alterations in the distal nephron after acute proximal tubular injury in the rat. *Lab Invest* 70:479–493, 1994
11. Honda N, Hishida A, Ikuma K, Yonemura K: Acquired resistance to acute renal failure. *Kidney Int* 31:1233–1238, 1987
12. Ikuma K, Honda N, Hishida A, Nagase M: Loss of glomerular responses to vasoconstrictor agents in rabbits recovering from ARF. *Kidney Int* 30:836–841, 1986
13. Ikuma K, Honda N, Yonemura K, Ohishi K, Hishida A, Nagase M: Glomerular refractoriness to contractile stimuli in rabbits recovering from ischemic acute renal failure. *Nephron* 48:306–309, 1988
14. Honda N, Yonemura K, Yamada M, Hishida A, Ikuma K: Acquired resistance in acute renal failure, in *Acute Renal Failure*, edited by Solez K, Racusen LC, New York, Marcel Dekker, Inc., 1991, pp 457–465
15. Furuya R, Kumagai H, Hishida A: Acquired resistance to rechallenge injury with uranyl acetate in LLC-PK1 cells. *J Lab Clin Med* 129:347–355, 1997
16. Mizuno S, Fujita K, Furuya R, Hishida A, Ito H, Tashima Y, Kumagai K: Association of HSP73 with the acquired resistance to uranyl acetate-induced acute renal failure. *Toxicology* 117:183–191, 1997
17. Yonemura K: Acquired resistance to acute renal failure in ischemic and uranium-induced renal failure of rabbits. *Jpn J Nephrol* 28:1221–1227, 1986
18. Mailhos C, Howard MK, Latchman DS: Heat shock proteins neuronal cells from programmed cell death by apoptosis. *Neuroscience* 55:621–627, 1993
19. Hockenbery D, Nuñez G, Milliman C, Schreiber DS, Korsmeyer SJ: Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. *Nature* 348:334–336, 1990
20. Korsmeyer S: Bcl-2 initiates a new category of oncogenes: Regulators of cell death. *Blood* 80:879–886, 1992
21. Oltavi ZN, Milliman CL, Korsmeyer SJ: Bcl-2 heterodimerizes in vivo with conserved homolog, Bax, that accelerates programmed cell death. *Cell* 74:609–619, 1993
22. Smith CA, Williams GT, Kingston R, Jenkinson EJ, Owen JJT: Antibodies to CD3/T-cell receptor complex induce death by apoptosis in immature T cells in thymic cultures. *Nature* 337:181–184, 1989
23. Gavrieli Y, Sherman Y, Ben-Sasson SA: Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. *J Cell Biol* 119:493–501, 1992
24. Sugihara H, Hattori T, Fukuda M: Immunohistochemical detection of bromodeoxyuridine in formalin-fixed tissues. *Histochemistry* 85:193–195, 1986
25. Yoshimura S, Banno Y, Nakashima S, Takenaka K, Sakai H, Nishimura Y, Sakai N, Shimizu S, Eguchi Y, Tsujimoto Y, Nozawa Y: Ceramide formation leads to caspase-3 activation during hypoxic PC12 cell death. *J Biol Chem* 273:6921–6927, 1998
26. Kajstura J, Cheng W, Reiss K, Clark WA, Sonnenblick EH, Krajewski S, Reed JC, Olivetti G, Anversa P: Apoptotic and necrotic myocyte cell deaths are independent contributing variables of infarct size in rats. *Lab Invest* 74:86–107, 1996
27. Lieberthal W, Menza SA, Levine JS: Graded ATP depletion can cause necrosis or apoptosis of cultured mouse proximal tubular cells. *Am J Physiol* 274:F315–F327, 1998
28. Miyachi K, Fritzler MJ, Tan EM: Autoantibody to a nuclear antigen in proliferating cells. *J Immunol* 121:2228–2234, 1978
29. Mccormick D, Hall PA: The complexities of proliferating cell nuclear antigen. *Histopathology* 21:591–594, 1992
30. Barnes DM, Gillett CE: Determination of cell proliferation. *J Clin Pathol* 48:M2–M5, 1995
31. Gillet CE, Barnes DM, Camplejohn RS: Comparison of three cell cycle associated antigens as markers of proliferative activity and prognosis in breast carcinoma. *J Clin Pathol* 46:1126–1128, 1993
32. Gratzner HG: Monoclonal antibody to 5-bromo-and-5-iododeoxyuridine: A new reagent for detection of DNA replication. *Science* 218:474–475, 1982
33. Petit MA, Bedale W, Osipiuk J, Lu C, Rajagopalan M, McInerney P, Goodman MF, Echols H: Sequential folding of UmuC by the hsp70 and hsp60 chaperone complexes of *Escherichia coli*. *J Biol Chem* 269:23824–23829, 1994